THE INFLUENCE OF DIETHYLSTILBESTROL ON METABOLIC ACTIVITIES OF AEROBACTER AEROGENES

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CHAPTER I

INTRODUCTION

The concept that estrogens and androgens are capable of functioning as metabolic regulators is comparatively new, even though the synthetic estrogen stilbestrol[1] has been employed as a growth-stimulating compound for several years. Oral administration or subcutaneous implants of stilbestrol result in an increased rate of gain in feeder lambs and cattle (1, 5). Experimental results have indicated that stilbestrol inhibits succinoxidase activity in various tissues (4, 8, 11, 12, 14), and stimulates proteinase activity in the pituitary tissue of rats (13). However, it does not seem plausible that these two effects could account for the total alteration of metabolism observed in experimental animals.

Since ruminant digestion involves the metabolic activity of a complex microflora, it is possible that stilbestrol might exert an influence by altering the population or the metabolism of these organisms. Stimulation of cellulose digestion by ovine rumen microorganisms has been reported both *in vitro* and *in vivo*, but test animals could not tolerate the levels of stilbestrol required to produce the *in vivo* stimulation (2). These results suggest that the sites which are sensitive to stilbestrol activity are present both in metabolic mechanisms of the animal and those of the rumen microflora.

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[1]Diethylstilbestrol; 4,4'-dihydroxy-α,β-diethylstilbene.

Additional information concerning the effect of stilbestrol on microorganisms is limited. Brownlee et al. (3) reported that stilbestrol-type compounds were bactericidal against gram-positive staphylococcus and streptococcus. On the other hand, San Clemente and MacKenzie (16) have suggested that stilbestrol exerts only a bacteriostatic effect on a rather wide range of gram-positive organisms. These authors also showed that gram-negative organisms were not appreciably affected by stilbestrol. Salmony (15) and Schacter (17, 18, 19) have reported that stilbestrol stimulates the endogenous respiration of yeast, but that the oxidation of a number of substrates by this organism is inhibited.

Stilbestrol is incorporated extensively in feedstuffs to facilitate rate of gain in animals and therefore is important in agricultural economics. Since reports concerning the effects of this compound are inconclusive and tend to be conflicting in several instances, additional knowledge of the mechanism(s) involved in its activity is desirable. The studies reported herein were undertaken to elucidate some of the basic effects exerted by stilbestrol on metabolic processes of Aerobacter aerogenes, in order that a better understanding of its primary function(s) might be gained.

CHAPTER II

MATERIALS AND METHODS

Methods included in this section are of a general nature. Deviations from these procedures are appropriately noted in the text.

Test organism.

A laboratory stock culture of Aerobacter aerogenes was employed in all studies. This organism was maintained on nutrient agar slants. Cultures were incubated for 24 hours at 37° C then refrigerated between transfers.

Synthetic medium.

The synthetic medium employed in these studies was composed of the following: NaCl, 0.20 g.; KH_2PO_4, 0.32 g.; K_2HPO_4, 0.42 g.; and NH_4Cl, 0.20 g. dissolved in 100 ml. of distilled water. After sterilization, 0.1 ml. of a mineral salts solution was added. Solid medium was prepared by adding agar (2%) as the solidifying agent.

Mineral salts solution.

The mineral salts solution was sterilized separately and added to the basal medium just prior to use. The following compounds were added to 100 ml. of distilled water to make up this solution: $MgSO_4 \cdot 7H_2O$, 0.5 g.; $MnSO_4$, 0.1 g.; $FeCl_3$, 1.0 g.; and $CaCl_2$, 0.5 g.

Manometric studies.

Respirometer studies were performed employing standard techniques (22).

Experiments were conducted at 37° C, using air as the gas phase. Absorption of carbon dioxide was accomplished by placing 0.2 ml. of 20 per cent potassium hydroxide and a strip of fluted filter paper in the center well of the Warburg flask. Substrates were placed in the sidearms and the cell suspension in the reaction compartment of the flask.

Cell counts.

Total cell counts were made from aliquots of the respective test systems. Aliquots were removed, diluted appropriately, and plated on nutrient agar. The double layer plating method was used and consisted of pipetting the diluted cell suspension into a plate containing a thin layer of agar, adding more agar, and mixing before solidification. To insure accuracy three dilutions were plated in triplicate. The colonies were counted after incubation for 24 hours at 37° C.

Ammonium sulfate.

Reagent grade ammonium sulfate was recrystallized by adding 2.0 g. of (ethylenedinitrilo)tetraacetic acid (versene) per liter of saturated ammonium sulfate, adjusting the pH to slightly basic conditions (7.4-7.8) with ammonium hydroxide then adding additional ammonium sulfate to obtain super-saturation at 100° C. On cooling, ammonium sulfate crystals precipitated. These were removed by filtration, dried, and employed in all ammonium sulfate fractionations.

Protamine sulfate.

Protamine sulfate (1%) was dissolved in 0.03 M phosphate buffer and the pH adjusted to 7.6. This solution was used in all protamine sulfate fractionations.

Fractionated protein solutions.

A. aerogenes protein fractions were prepared by grinding the cells in a Virtis homogenizer with glass beads, rinsing the beads with three volumes of 0.01 M tris buffer of pH 7.0, and centrifuging the wash for 30 minutes at 10,000 x g. The supernatant was then centrifuged at 20,000 x g. for 1 hour after which the supernatant was decanted and fractionated. Protamine sulfate was added with mixing until a rather dense stringy white precipitate formed. This precipitate was removed by centrifugation. Ammonium sulfate was then added to the supernatant to 70 per cent saturation and mixed for 15 minutes. The precipitate was removed by centrifugation, resuspended in a small volume of the tris buffer, and dialysed against distilled water. The dialysate was further fractionated with ammonium sulfate as desired by following the same procedure.

Hydrogen cyanide solution.

A 0.01 M solution of hydrogen cyanide was prepared by adding 0.8 ml. of 1.0 N hydrochloric acid to 66 mg. of potassium cyanide in 99.2 ml. of distilled water. The pH of this solution was approximately 8.0.

Succinic dehydrogenase assay.

The activity of succinic dehydrogenase was followed employing the phenazine methosulfate assay (20) by measuring oxygen uptake manometrically. The protein solution, 0.5 ml. of 0.3 M phosphate buffer of pH 7.6, 0.1 ml. of 0.01 N sodium hydroxide or stilbestrol (20 ug) in sodium hydroxide and succinate were placed in the reaction compartment of the Warburg flask. Phenazine methosulfate was placed in one sidearm and 0.3 ml. of hydrogen cyanide solution or catalase (1.75 mg., yeast) and 0.05 ml. of ethanol (95%) were pipetted into the other sidearm. Distilled

water was added to bring the total volume to 3.0 ml. After an equilibration period of 10 minutes at 37° C, the reactants in the sidearms were dumped and oxygen consumption followed. The optimum concentration of phenazine methosulfate was determined for each protein preparation.

CHAPTER III

EXPERIMENTAL AND RESULTS

The Effect of Stilbestrol on the Growth of A. aerogenes.

Since stilbestrol increases the rate of gain in ruminants and some species of fowls, and has been reported to exert various influences on microorganisms, it was desirable to elucidate mechanisms involved in the activities of this estrogen. Studies were conducted to determine if stilbestrol influenced the growth of A. aerogenes. A mono-, di-, tri-, and polysaccharide were employed individually as sole sources of carbon and energy for aerobic growth.

The cells to be used as the inoculum were grown in synthetic medium with 0.1 per cent glucose as the carbon source by incubating for 20 hours at 37° C with constant shaking. An aliquot was removed, washed three times with 0.02 M phosphate buffer of pH 7.0, and resuspended in buffer to give 10 per cent transmittance at 420 mu in a Spectronic 20 colorimeter. One-half ml. of this standardized cell suspension was used as the inoculum.

The carbohydrates were heat sterilized separately and added to the synthetic medium just prior to use. The substrates were present in a final concentration of 0.1 per cent, with the exception of starch which was employed in a concentration of 0.05 per cent. Stilbestrol was added directly to the synthetic medium in a final concentration of 1.0 mg per ml.

Calibrated tubes, containing 9.5 ml. of synthetic medium plus carbohydrate and 0.5 ml. of inoculum, were incubated at 37° C. Growth was

followed by measuring per cent transmittance at 420 mu. Since stilbestrol is only slightly soluble in aqueous solution, readings were made both befor and after shaking to facilitate accurate determinations. Controls containing the inoculum with substrate and inoculum with stilbestrol were included in all experiments.

The effect of stilbestrol on the growth of A. aerogenes was determined using glucose, maltose, raffinose, and starch as substrates. Experimental controls, in which the carbohydrate was omitted and stilbestrol was present, showed that this organism is not capable of utilizing stilbestrol as a source of carbon and energy. Additional results indicated that the estrogen did not alter the growth of A. aerogenes significantly when either glucose, maltose, or raffinose was utilized as a source of energy, since the change in per cent transmittance in the stilbestrol containing tubes did not differ appreciably from that in the substrate controls. This observation was substantiated by results of viable cell counts, in which aliquots were removed from the test systems, plated on nutrient agar, and colonies counted after incubation. Viable counts from experiments employing glucose, maltose, and raffinose are presented in the Appendix.

A stimulation or enhancement of growth was observed when starch was employed as the substrate. The data indicated that the lag period was considerably shorter in the presence of stilbestrol than in the starch control. This observation was confirmed by viable cell counts which showed that the number of viable cells increased at a more rapid rate in the stilbestrol containing system than in the starch control system. Results of the plate counts are incorporated in the Appendix.

To determine if the concentration of stilbestrol could be related

to the enhancement of growth, studies were performed in which the concentration of stilbestrol was varied. Results of this experimentation, shown in Figure 1, indicated that the degree of stimulation was dependent on the concentration of stilbestrol, since the initiation of growth occurred earliest in the highest concentration of stilbestrol (5.0 mg per ml.), followed by growth in the 1.0 mg per ml., 0.1 mg per ml., and 0.0 mg per ml. respectively. Aliquots from this test system were removed from the tubes at various time intervals, placed directly into Warburg flasks, and oxygen uptake measured following a temperature equilibration period of 30 minutes. The results from aliquots removed at 43 hours are presented in Figure 2. In general, it was observed that oxygen consumption was greater in the stilbestrol containing systems. The increased oxygen consumption per unit time in stilbestrol vessels may be accounted for by the increased number of cells since growth originated earlier in these systems. Since additional starch was not added to the Warburg flask, the substrate concentration may have been limiting in the 5.0 and 1.0 mg stilbestrol vessels.

Additional experiments were performed in which the growth medium of the inoculum was varied. The cells used as the inoculum were grown in synthetic medium plus starch, then 1.0 ml. of the actively growing culture was transferred to each of two tubes, one containing starch medium, the other containing starch medium plus stilbestrol. The per cent transmittance of the starch control and stilbestrol containing tubes were: 74 and 72 at 2 hours; 66 and 67 at 4 hours; 65 and 63 at 6 hours; 47 and 49 at 16 hours. These results showed that growth-stimulation did not occur under these conditions, since growth in the stilbestrol containing tube did not differ appreciably from that in the starch control.

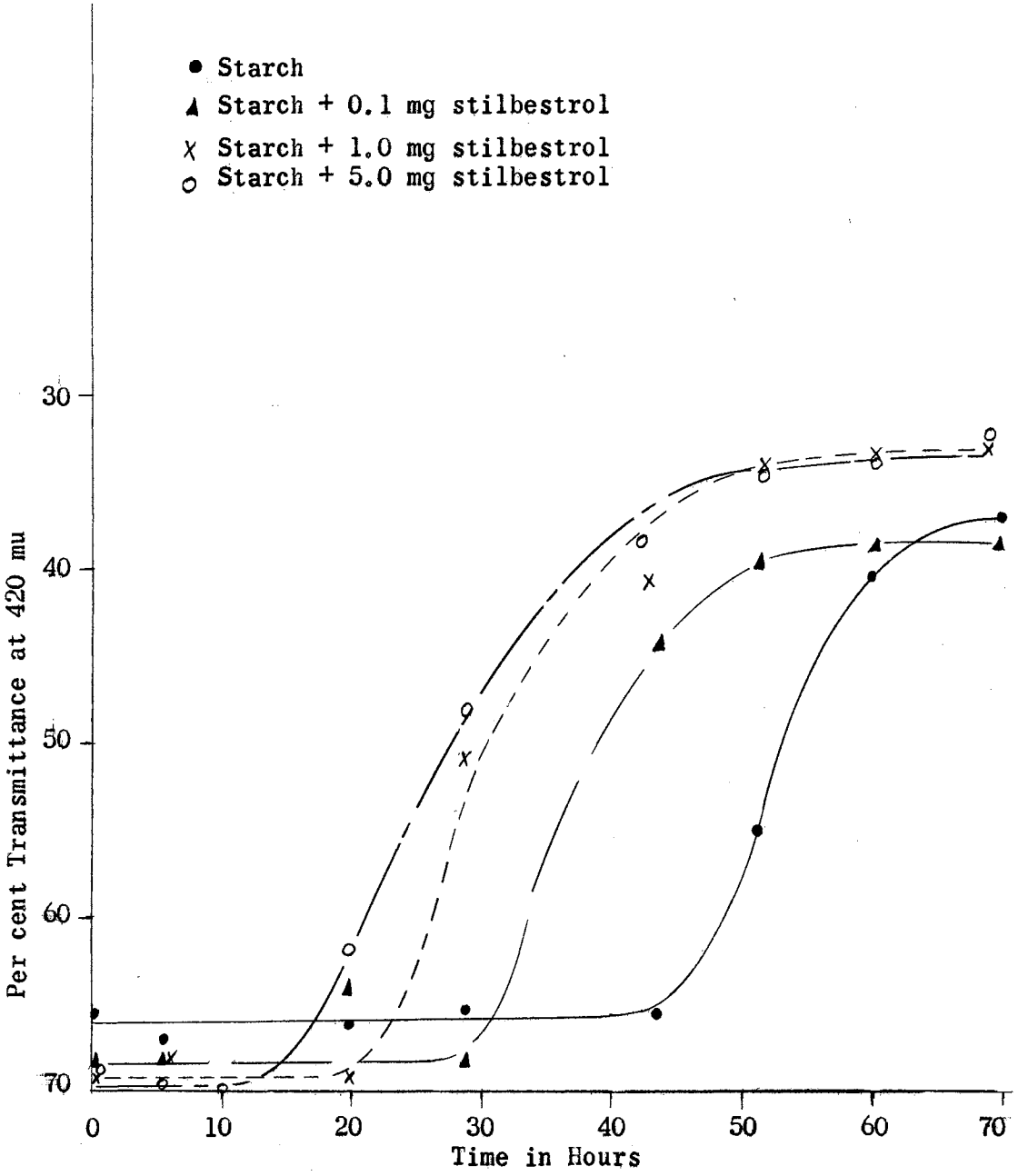

Figure 1. The Growth of A. aerogenes in Synthetic Starch Medium with Varying Concentrations of Stilbestrol

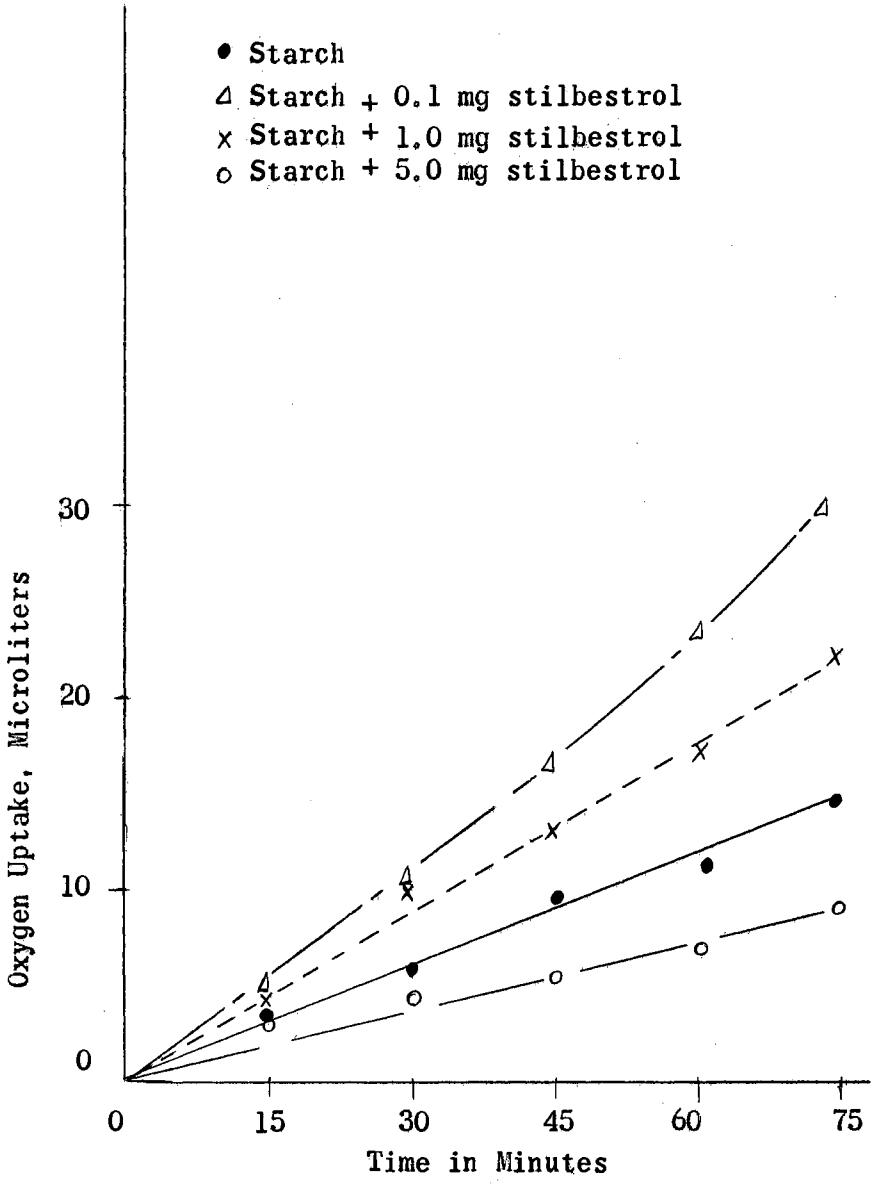

Figure 2. The Oxidation of Starch by A. aerogenes with Varying Concentrations of Stilbestrol

The data suggest that the stilbestrol-stimulation of the growth of A. aerogenes in the synthetic starch medium is due to a stimulatory effect on the enzyme responsible for starch hydrolysis, either by stimulating the enzymic reaction or by stimulating enzyme formation. The latter suggestion seems more reasonable since growth of the cells which were actively metabolizing starch was not influenced by the addition of stilbestrol. Results which show that the lag period was shortened when stilbestrol was present during the "adaptation" period also support this view, as it would seem that the initiation of growth would occur at the same time in the presence or absence of stilbestrol if the enzyme formation were not being stimulated.

The Effect of Stilbestrol on Substrate Oxidation

It has been reported that stilbestrol influences the rate of oxidation of various substrates by animal tissues (4, 8, 9, 11, 12, 13, 14, 23) and yeast cells (15, 17, 18, 19). In addition, results obtained in the experimentation reported in the previous section indicated that stilbestrol influences the oxidative dissimilation of starch by A. aerogenes. To determine if this estrogen is capable of influencing the oxidation of other compounds, manometric experiments were performed in which a number of carbohydrates and metabolic intermediates were employed as substrates.

A. aerogenes cells were grown on nutrient agar for 20-24 hours at 37° C, harvested, washed three times with 0.008 M phosphate buffer of pH 7.0, and resuspended in buffer. Stilbestrol was suspended in 0.01 N sodium hydroxide and allowed to stand with intermittent mixing for 1 hour before being incorporated in the test system in a final concentration of 0.36 mg per ml. The substrates were dissolved in 0.008 M phosphate buffer and the pH adjusted to 7.0.

The cell suspension and stilbestrol were placed in the reaction compartment of the Warburg flask, and the substrate was pipetted into the sidearm. After a temperature equilibration period of at least 20 minutes, the substrate was dumped into the flasks. The final pH of the flask contents was 7.8–8.0. Substrate and stilbestrol controls were run concurrently.

The data obtained in these studies indicate that stilbestrol inhibits the oxidation of maltose, fructose, glucose, glutamate, oxalacetate, succinate, and pyruvate. The inhibition appeared to be on the rate of oxidation of these compounds rather than the extent of oxidation, since the total oxygen consumed in the presence and absence of stilbestrol was essentially the same. The estrogen did not affect endogenous respiration significantly.

In opposition to the inhibitory effect was the observation that stilbestrol stimulated the oxidation of raffinose (Figure 3). Again, the effect appeared to be on the rate of oxidation rather than on the extent, as the total oxygen consumption in the stilbestrol containing system did not differ appreciably from that of the raffinose control.

The observations which indicated that glutamate oxidation is inhibited, and that stilbestrol did not influence the endogenous respiration of A. aerogenes, are in opposition to reports that stilbestrol does not affect yeast cell respiration in the presence of glutamate (15), but increases endogenous respiration (15, 18). It is quite possible that the differences in results may be due to varied experimental conditions in the differing biological systems employed in these studies. In order to define the conditions under which stilbestrol influences the metabolism of A. aerogenes, additional experimentation was undertaken.

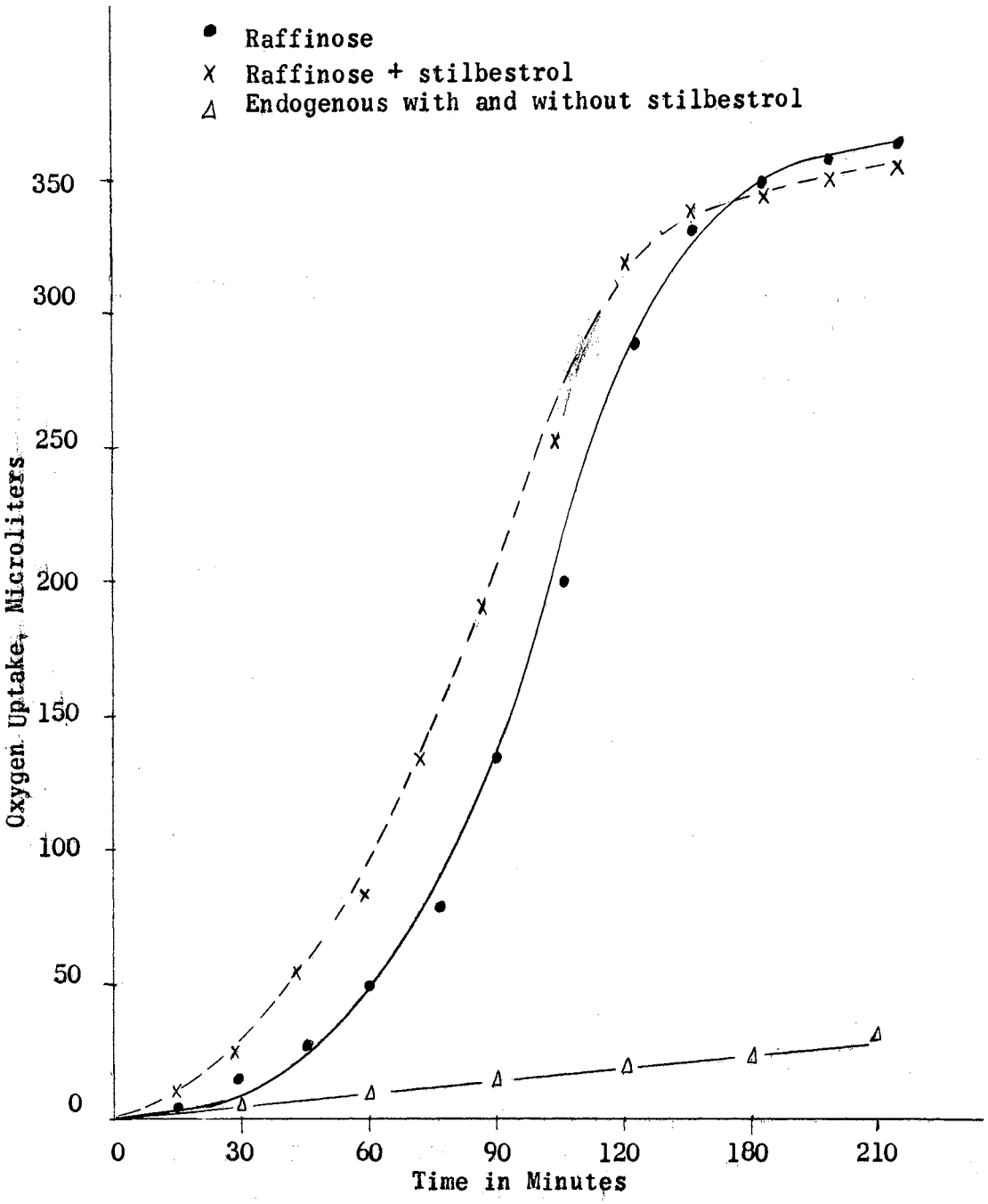

Figure 3. The Oxidation of Raffinose by A. aerogenes in the Presence and Absence of Stilbestrol

Factors Influencing the Effects of Stilbestrol

It has been suggested that the discrepancies in experimental results which have been reported concerning the effects of stilbestrol on animal tissues have been due to varied experimental conditions (14). Deviations in manometric procedures were made in order to more clearly define the conditions under which stilbestrol affects the metabolism of A. aerogenes.

To ascertain if preexposure of the cells to the estrogen influenced the observed effects, identical sets of flasks were prepared and the substrate was dumped in one set of vessels at intervals of 0, 1, 2, 3, and 4 hours respectively. Results indicated that the inhibition of oxygen uptake may be correlated to the length of time the cells were exposed to stilbestrol prior to dumping the substrate, since the degree of inhibition increased as the length of time of preexposure increased. This preexposure effect was observed when glucose, maltose, fructose, succinate, or pyruvate oxidation was followed. Figure 4 presents the results obtained when succinate was employed as the substrate, showing that the degree of inhibition was greater after 3 hours of preexposure to stilbestrol than at 0 hours. The stimulation of raffinose oxidation was also found to be dependent on preexposure of the cells to stilbestrol, as the initiation of oxidation occurred earlier with increasing lengths of exposure time (Figure 5). These observations are in accord with results obtained when mammalian tissues were exposed to stilbestrol for increasing time periods (23).

To determine what effect hydrogen ion concentration might have on the stilbestrol-inhibition of glucose oxidation, the pH of the flask contents were varied to include the range of 7.0 to 8.5. Data from this study, shown in Table I, indicated that stilbestrol exerts little, if any,

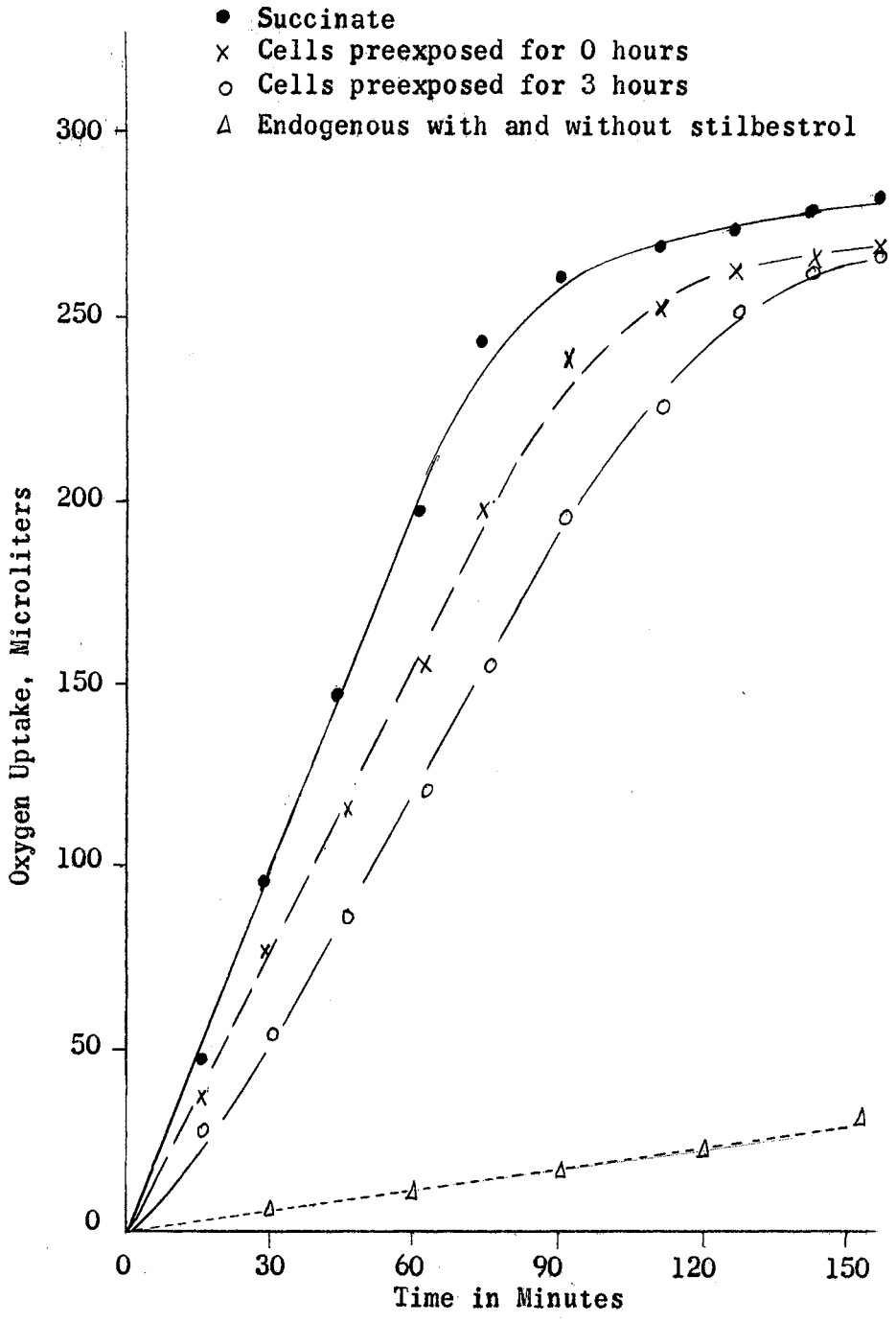

Figure 4. The Oxidation of Succinate by A. aerogenes after Preexposure of the Cells to Stilbesterol for Varying Time Periods

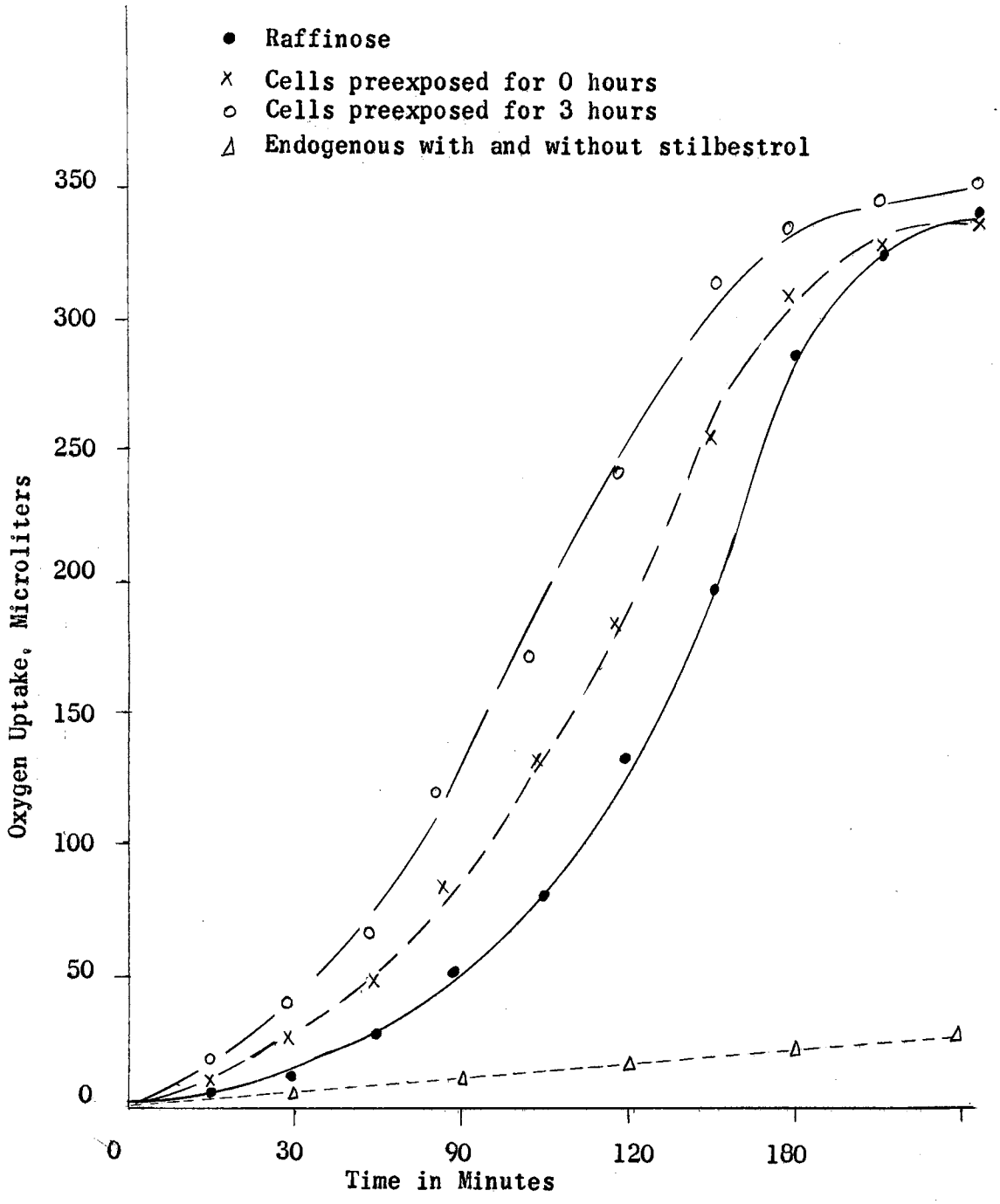

Figure 5. The Oxidation of Raffinose by A. aerogenes after Pre-exposure of the Cells to Stilbestrol for Varying Time Periods

TABLE I

THE OXIDATION OF GLUCOSE BY A. AEROGENES IN THE PRESENCE AND ABSENCE OF STILBESTROL AT VARYING INITIAL pH

Time in Minutes	Oxygen Uptake in Microliters							
	pH 7.1		pH 7.4		pH 7.9		pH 8.5	
	Control	Stilbestrol	Control	Stilbestrol	Control	Stilbestrol	Control	Stilbestrol
15	125	111	102	89	117	82	120	83
25	168	159	145	139	168	133	168	126
35	212	195	181	176	211	180	214	166
45	253	232	213	213	253	217	247	205
55	274	253	225	230	268	253	255	242
65	289	271	235	243	273	275	260	248

effect at pH levels below 7.5, as the rate of glucose oxidation in the presence of stilbestrol was essentially the same as in the glucose control. However, above this pH the inhibition in the rate of oxygen uptake was reproducible and appears to be enhanced as the pH is shifted toward more basic conditions. It is possible that changing the pH results in an alteration of the permeability barrier, thus accounting for the observed effects. However, this does not seem plausible since the shift in pH did not influence the rate of substrate oxidation in the controls. Since stilbestrol is more soluble in slightly basic solutions than in neutral solutions, it is suggested that the increased effect at higher pH levels may be due to more stilbestrol being in solution, thus increasing its concentration at the sensitive sites. Studies were also conducted to determine if the observed effects could be a function of stilbestrol concentration. The oxidation of glucose and raffinose were studied with varying estrogen concentrations (Figure 6). Results showed that the inhibition of glucose oxidation is related to stilbestrol concentration, as the rate of oxidation decreased with increasing inhibitor concentration. Stilbestrol concentration is also functional in the stimulation of raffinose oxidation, since increasing estrogen concentrations enhanced oxygen uptake. Results also indicated that there may be two levels in the stimulatory effect.

These studies, concerning the conditions under which stilbestrol influences oxidations by A. aerogenes, indicated that the degree of inhibition could be correlated with estrogen concentration, preexposure of the cells, and the pH of the reaction system. These factors also influence the stimulation of raffinose oxidation. Results suggest that these variables may exert their effect by allowing stilbestrol to reach the sensitive

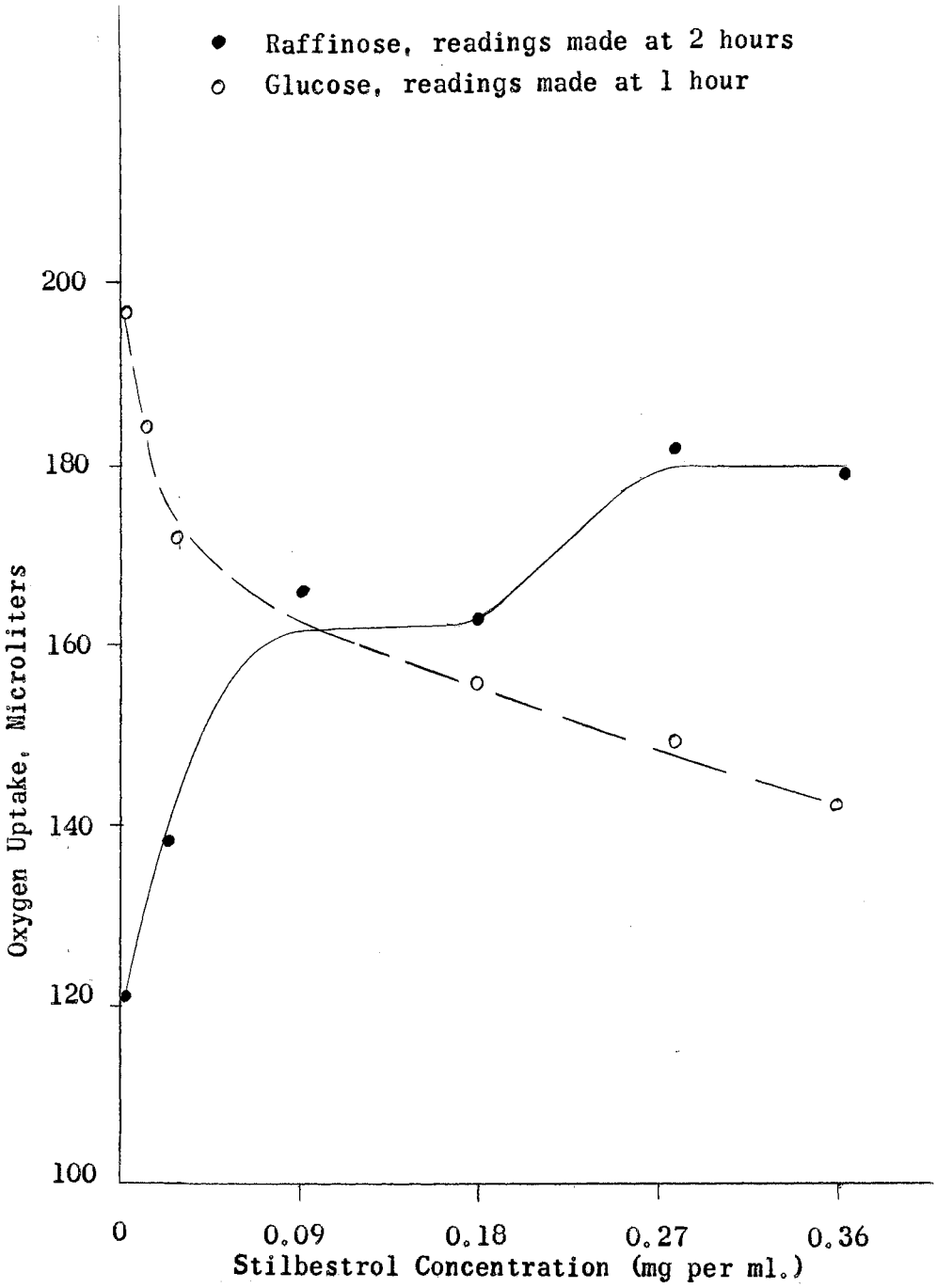

Figure 6. The Oxidation of Glucose and Raffinose by A. aerogenes in the Presence of Varying Concentrations of Stilbestrol

site(s) in a higher concentration thereby resulting in enhanced effects. However, it is also possible that stilbestrol is influencing cell-wall mechanisms which may involve the active transport of substrate across the cell membrane.

Stilbestrol Inhibitions in Intact and Ruptured Cells

In order to ascertain if the inhibitory activity of stilbestrol was due to its influencing cell-wall mechanisms or intracellular enzyme systems, studies were conducted which allowed a comparison of the effect of stilbestrol on intact cells and cell-free extracts of A. aerogenes. Since stilbestrol stimulates the growth of this organism in starch and is also capable of enhancing raffinose oxidation, it did not seem likely that the inhibitory effect observed in other substrate oxidations was due to bactericidal activity by the estrogen. However, to clarify this possibility, duplicate flasks were prepared and viable counts were made from one set of flasks while oxygen consumption was measured in the other. Colony counts made from the test systems at various time intervals showed that this estrogen does not influence the number of viable cells (Table II). These data indicate that the decreased rate of oxygen consumption is not the result of bactericidal activity by the estrogen since the number of viable cells in the stilbestrol containing system did not differ from the number in the controls.

Experiments were also performed in which substrate oxidation was studied after allowing the cells to stand in the presence and absence of stilbestrol for as long as 5 hours. Following standing the cells were washed twice, standardized to the same turbidity, and glucose oxidation was measured in the presence and absence of stilbestrol. Results from this experimentation, shown in Table III, indicated that soaking the

TABLE II

TOTAL NUMBER OF VIABLE CELLS FOLLOWING EXPOSURE OF A. AEROGENES TO STILBESTROL FOR VARYING TIME PERIODS

Expt. No.	Number of Viable Organisms per ml.						
	Initial Count	Time of Exposure					
		1 Hour		2 Hour		3 Hour	
		Control	Stilbestrol	Control	Stilbestrol	Control	Stilbestrol
	$\times 10^8$	$\times 10^8$		$\times 10^8$		$\times 10^8$	
1	53	40	61				
2	71	60	82				
3	46	41	43	48	26		
4	108	109	96	112	124		
5	113	117	98	105	89	98	101

TABLE III

THE OXIDATION OF GLUCOSE IN THE PRESENCE AND ABSENCE OF STILBESTROL AFTER SOAKING A. AEROGENES CELLS IN THE PRESENCE AND ABSENCE OF STILBESTROL

Time in Minutes	Oxygen Uptake in Microliters			
	Untreated and Washed		Stilbestrol Treated and Washed	
	Control	Stilbestrol	Control	Stilbestrol
10	85	73	90	74
20	185	175	191	163
30	245	233	256	222
40	306	293	296	252
50	345	328	311	266
60	350	334	314	271

cells in stilbestrol did not influence their ability to oxidize glucose after the stilbestrol had been removed by washing, since the rate of oxygen uptake by estrogen-treated and washed cells was nearly identical to that of cells that had not been exposed to stilbestrol but had undergone the same treatment otherwise. In the presence of stilbestrol the rate of oxygen consumption was inhibited in both the treated and untreated cells. These data suggest that the estrogen must be present to exert its influence and does not do irreversible damage to the cell.

Additional experiments were then conducted to determine if stilbestrol would inhibit enzymic reactions by extracts of A. aerogenes. Crude cell-free extracts were prepared by grinding the cells with glass beads in a Virtis homogenizer and then gently rinsing the beads with a small volume of 0.008 M phosphate buffer of pH 7.0. The wash was decanted, centrifuged at 5,000 x g for 30 minutes, and the resulting supernatant was used as the crude extract.

Substrate oxidation was measured by following the rate of reduction of either methylene blue or brilliant cresyl blue in Thunberg tubes. All components except the substrates were placed in the tubes. The substrate was placed in the sidearm. The tubes were then subjected to a vacuum for 5 minutes with continuous gentle tapping to aid evacuation. The substrate was dumped after a temperature equilibration period of 10 minutes. The concentrations of the reaction components per ml. were identical to those in the respirometer studies. The hydrogen carriers were employed in concentrations which were not inhibitory to the enzymic reactions, and varied for different crude extract preparations.

Results indicated that stilbestrol inhibits the rate of succinate, pyruvate, and glucose oxidation when either methylene blue or brilliant

cresyl blue are employed, since the rate of dye reduction in the presence of stilbestrol was markedly slower than in its absence. Additional experimentation with crude extracts confirmed the finding that the inhibition of succinate oxidation is dependent on the concentration of stilbestrol, since increasing the estrogen concentration resulted in a decreased rate of dye reduction. Results of experimentation in which the reduction of brilliant cresyl blue was followed visually during the oxidation of succinate are presented in Table IV showing that the inhibition in the rate of dye reduction is related to the concentration of stilbestrol.

It has been suggested that stilbestrol influences the enzyme systems in the cell membrane responsible for the "active transport" of glucose into the yeast cell (19). The data reported in this investigation indicate that oxidations by cell-free extracts of A. aerogenes are also inhibited by stilbestrol. These findings do not support the "active transport" hypothesis, since the inhibitions are observed in preparations in which cell-walls are no longer intact. The data also indicated that soaking the cells in stilbestrol did not result in irreparable damage to the cell membrane. It therefore seems feasible to postulate that this estrogen is not inhibiting by interfering with the mechanisms associated with membrane function in this organism.

Attempted Reversal of Stilbestrol Inhibition

Previous reports indicated that the stilbestrol-inhibition of the succinoxidase system in animal tissues could be reversed when either methylene blue (8) or brilliant cresyl blue (11) was incorporated in the system. An additional report states that the inhibition of malate oxidation by animal tissues was reversed by the inclusion of cytochrome c and

TABLE IV

THE REDUCTION OF BRILLIANT CRESYL BLUE DURING OXIDATION OF SUCCINATE BY A CRUDE CELL-FREE EXTRACT IN THE PRESENCE AND ABSENCE OF VARYING CONCENTRATIONS OF STILBESTROL

	Reduction of Brilliant Cresyl Blue				
Stilbestrol Concentration	1 hr.	2 hr.	3 hr.	5 hr.	7 hr.
Succinate	+	+	++	++++	++++
0.1 mg Stilbestrol		+	++	++++	++++
0.2 mg Stilbestrol			+	++	++++
0.3 mg Stilbestrol				+	+++
0.4 mg Stilbestrol					++

diphosphopyridine nucleotide (14). These workers suggested that cytochrome oxidase and succinic dehydrogenase are sensitive to the estrogen.

To elucidate the inhibitory activity of stilbestrol on substrate oxidation by A. aerogenes, various additives were incorporated into systems employing either suspensions of intact cells or crude cell-free extracts as the oxidizing agent. Manometric and Thunberg studies were performed in accordance with methods previously described.

Results from respirometer experiments utilizing cell suspensions in which a wide range of dye concentrations were employed, indicated that neither methylene blue nor brilliant cresyl blue reversed the stilbestrol-inhibition of the rate of oxidation of maltose, fructose, glucose, succinate, oxalacetate, or pyruvate. Representative results demonstrating that succinate oxidation is not reversed by methylene blue or brilliant cresyl blue are shown in Figure 7.

Other additives such as diphosphopyridine nucleotide (0.1 mg), adenosine triphosphate (0.2 mg), or cytochrome c (0.1-0.2 mg) were added to the test system with the electron acceptors. The results indicated that these additives did not overcome the inhibition. The suppression of oxygen uptake was also observed when the enzymic cofactors manganese, calcium, and magnesium were included in the system.

Thunberg experiments were performed with crude cell-free extracts. The rate of dye reduction was followed visually or by measuring the change in per cent light transmittance in a Spectronic 20 colorimeter. Results obtained from studies in which glucose, succinate, or pyruvate were employed as substrates indicated that stilbestrol suppressed the reduction of both methylene blue and brilliant cresyl blue. Additional investigation with crude extracts indicated that the estrogen inhibition was not

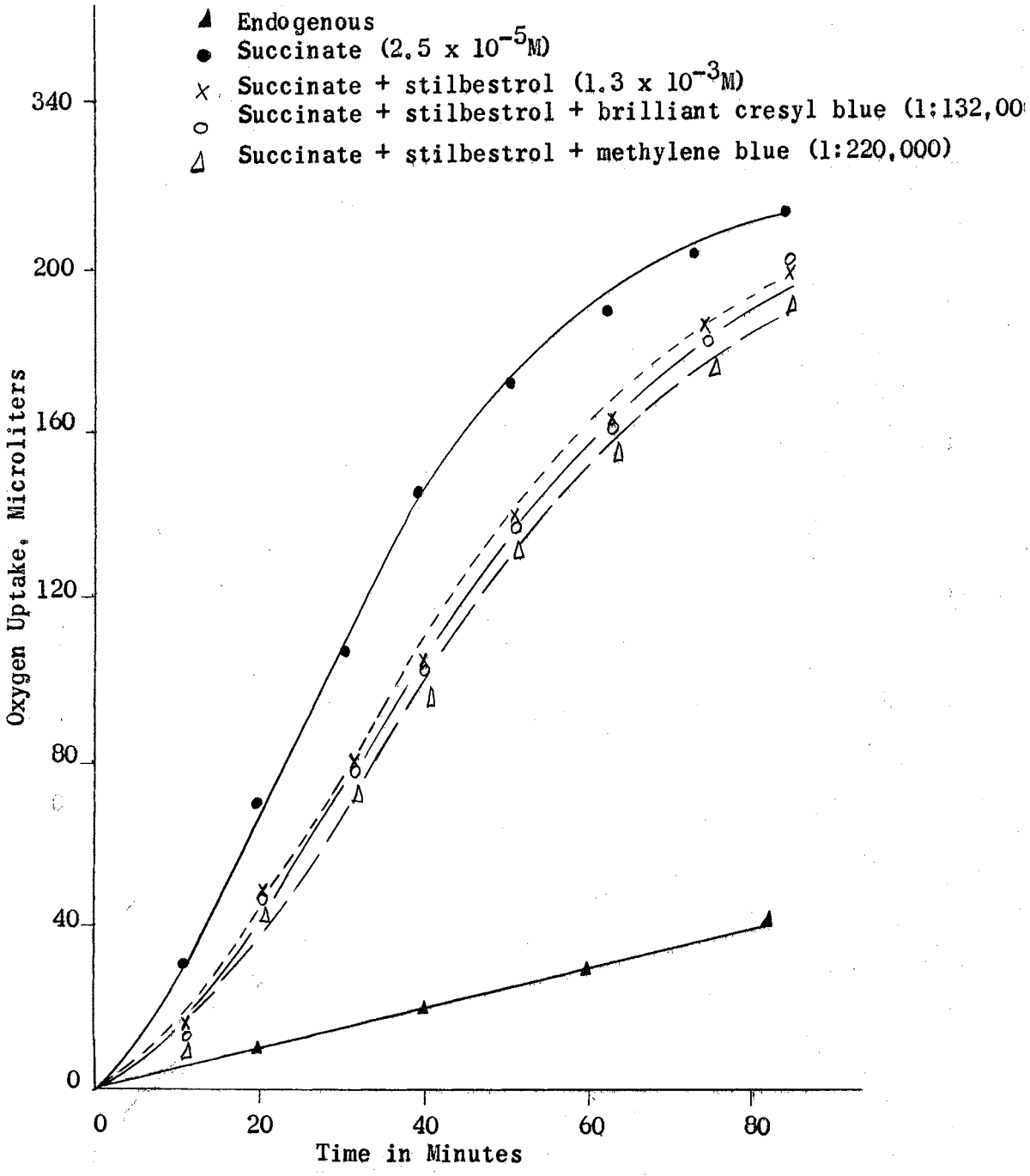

Figure 7. The Oxidation of Succinate by A. aerogenes in the Presence and Absence of Stilbestrol and Added Electron Acceptors

relieved by 2, 3, 5-triphenyl-tetrazolium chloride (Figure 8), cytochrome c, diphosphopyridine nucleotide, or riboflavin-5-phosphate. Similar studies indicated that yeast extract, vitamin-free casein hydrolysate, or casamino acid was also ineffective in reversing the effects of stilbestrol.

These data show that the inhibition of substrate oxidation could not be reversed by the addition of various organic metabolites, electron carriers, or metallic ions which function as enzymic cofactors. The observation that the inclusion of various additives did not reverse the inhibition could be explained on the basis that the additives were not penetrating the cell-wall, thus not reaching the estrogen sensitive sites. However, since cell-free systems were also inhibited by stilbestrol in the presence of the various additives, it seems more feasible to suggest that stilbestrol was interfering with oxidations by influencing intracellular metabolic mechanisms. Since substrate utilization involves a number of oxidative mechanisms, it was speculated that stilbestrol might be interfering with hydrogen transport systems, either by inhibiting the primary enzymes or by interfering with electron transport. The latter suggestion, however, does not seem likely since the incorporation of electron carriers did not overcome the inhibition. Nevertheless, the speculation has merit in view of reports that (a) enzyme systems from various biological sources do not respond to the same artificial electron carriers (10), (b) stilbestrol is capable of existing as a quinone (6, 7, 9), and (c) naturally occurring estrogens may be involved in certain transhydrogenation reactions (21).

Stilbestrol Activity in Hydrogen Transport Mechanisms

Experimental evidence indicated that stilbestrol influenced the oxidation of a number of substrates by A. aerogenes. Additional results

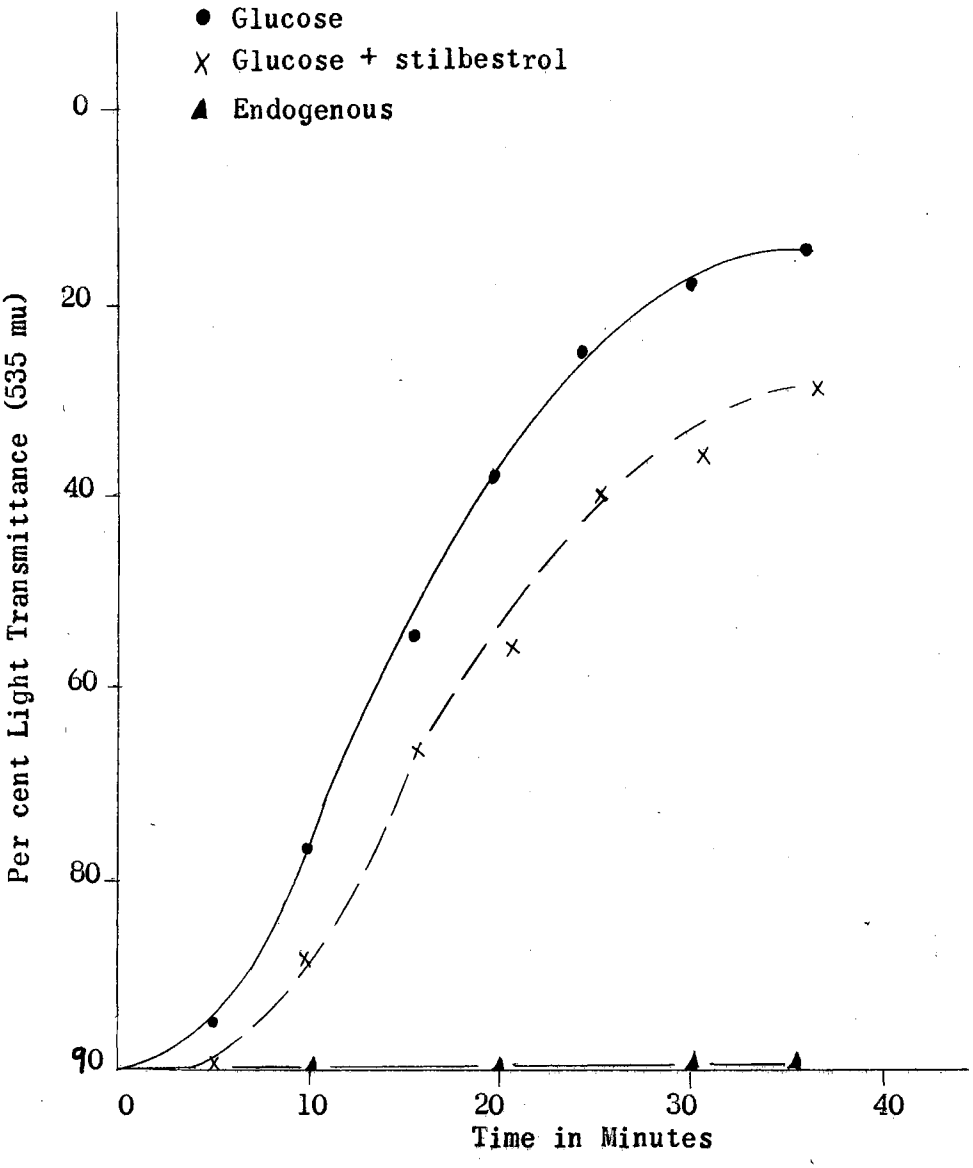

Figure 8. The Oxidation of Glucose by A. aerogenes in the Presence and Absence of Stilbestrol with 2, 3, 5-Triphenyl tetrazolium chloride

indicated that hydrogen transport mechanisms might be the sensitive sites in the inhibitory activity of the estrogen. In order to elucidate the influences that stilbestrol might exert on electron transport mechanisms, various cell-free extracts and protein fractions of A. aerogenes were prepared and employed in studies of oxidative reactions.

In recent years it has been reported that the naturally occurring estrogen, estradiol, functions in transhydrogenation reactions in certain animal tissues (21). The reactions reported to be involved in the assay of transhydrogenase activity are as follows:

$$\text{(I)} \quad \text{Substrate} + TPN^{+} \text{ ------ } \text{Product} + TPN\cdot H + H^{+}$$

$$\text{(II)} \quad DPN^{+} + TPN\cdot H \text{ ------ } DPN\cdot H + TPN^{+}$$

The transhydrogenation reaction (II) is reported to be estrogen dependent. To determine if the synthetic estrogen stilbestrol might be functioning as a catalyst in transhydrogenation reactions in A. aerogenes, experiments were performed in which the oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase and isocitrate by isocitric dehydrogenase were followed. Crude cell-free extracts demonstrated a high endogenous capacity to reduce pyridine nucleotides, therefore solutions obtained by ammonium sulfate fractionation were employed in these studies. The protein solution, manganese chloride (1 uM), tris buffer of pH 7.6 (200 uM), 0.1 ml. of 0.01 N sodium hydroxide or stilbestrol (20 ug) in sodium hydroxide, and distilled water to a final volume of 3.0 ml. was added in a 1 cm cuvette. TPN (0.1 uM), DPN (1.0 uM), and substrate were added at various time intervals. The change in optical density was measured at 340 mu in a Beckman DU spectrophotometer, in order to determine the reduction of pyridine nucleotides.

Results obtained in these studies, shown in Figure 9, indicate that

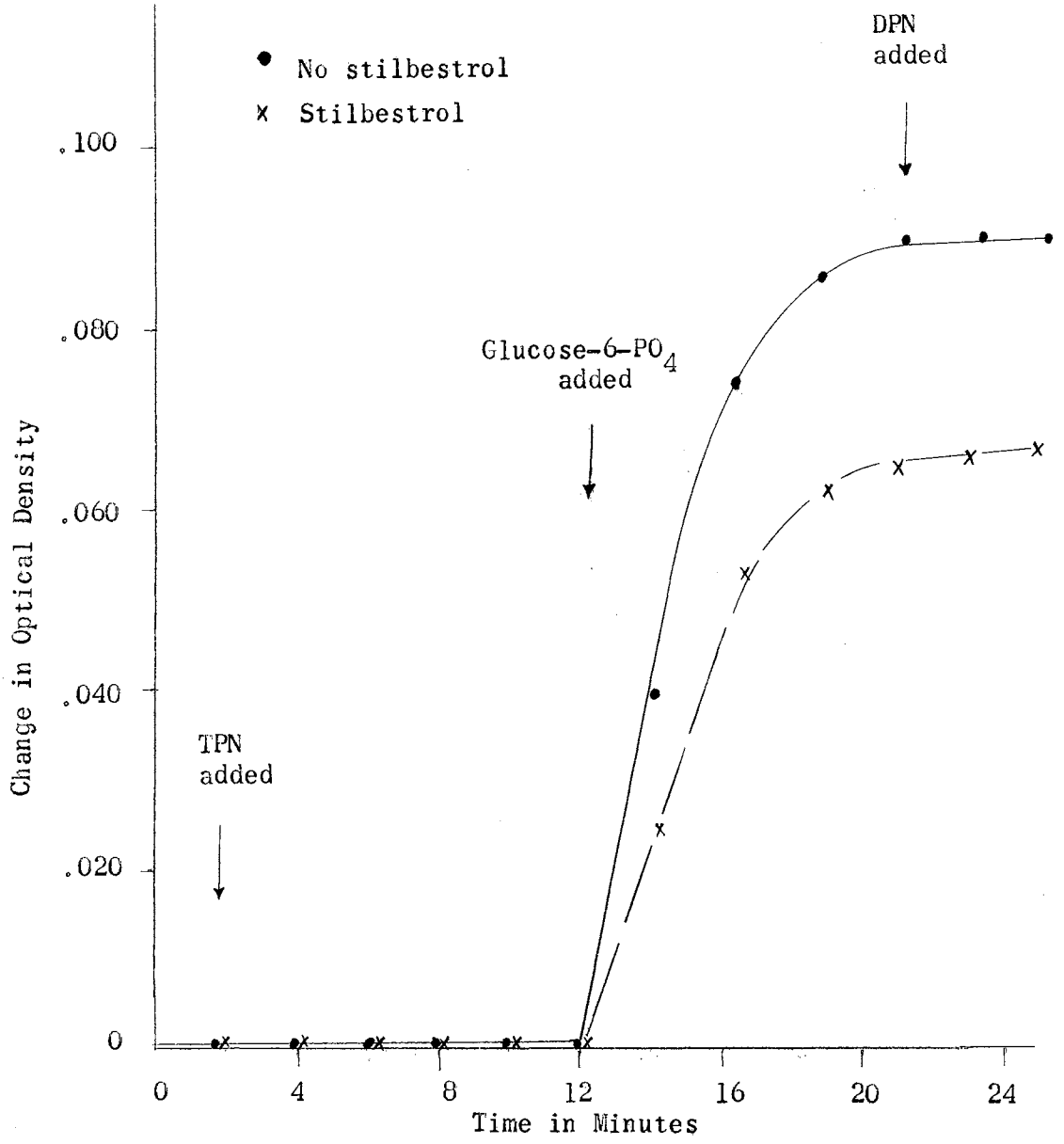

Figure 9. The Reduction of Pyridine Nucleotides by a 60-70 per cent Ammonium Sulfate Fraction of A. aerogenes in the Presence and Absence of Stilbestrol

stilbestrol apparently did not function in the transfer of electrons from from TPN·H to DPN^+ (reaction II), since after the reduction of TPN occurred (reaction I), a further increase in optical density was not observed on the addition of DPN. This finding is in agreement with results obtained from known estradiol sensitive transhydrogenation reactions in which stilbestrol did not influence transhydrogenase activity (21). The data did indicate that the reduction of TPN^+ (reaction I) was inhibited in the presence of stilbestrol when either glucose-6-phosphate or isocitrate were employed as the substrate. Figure 10 demonstrates typical results obtained when glucose-6-phosphate oxidation was followed in the presence and absence of various concentrations of stilbestrol. The data indicate that the inhibition in the rate of TPN^+ reduction may be correlated to the concentration of stilbestrol. However, the extent of inhibition of TPN·H formation appeared to be essentially the same in all concentrations of stilbestrol employed (0, 4, 20, and 40 mg respectively). Similar results were obtained when isocitrate was employed as the substrate. These findings suggest that stilbestrol might be serving as an electron carrier or an electron trap. It has been reported that stilbestrol is capable of forming an unstable quinone (7), therefore, the possibility that stilbestrol might be existing in a quinone form under the conditions of this experimentation was investigated.

A stilbene-quinone was first prepared by Euler and Adler (6) by using lead tetraacetate as the oxidizing agent for stilbestrol which could be dissolved in a variety of organic solvents. Fieser (7) prepared a stilbene-quinone in a solution of aqueous potassium molybdicyanide with an alcoholic buffer. More recently, Hochster and Quastel (9) while determining the effect of stilbestrol on substrate oxidations reported that a

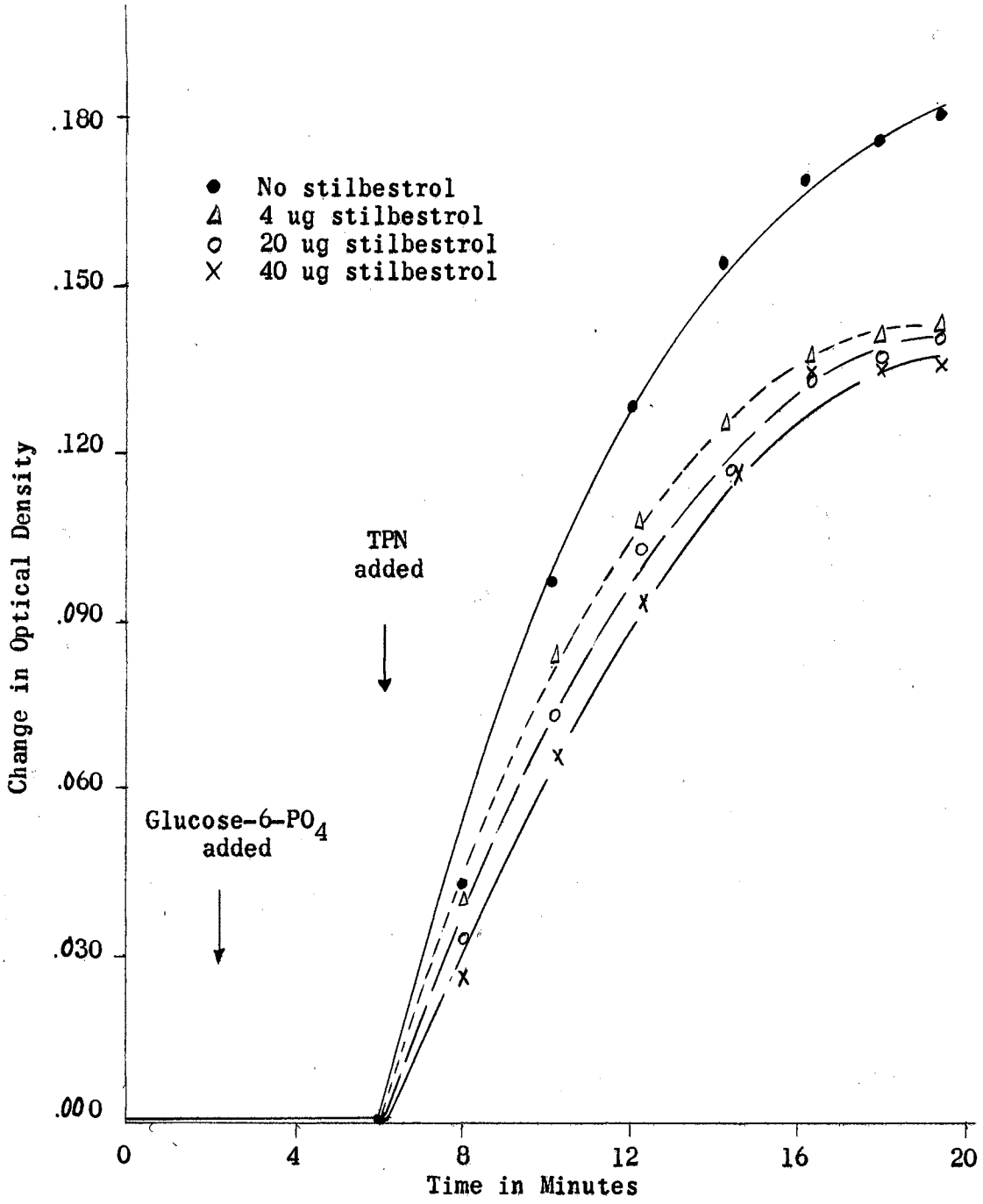

Figure 10. The Oxidation of Glucose-6-Phosphate by a 50-70 per cent Ammonium Sulfate Fraction of A. aerogenes in Varying Concentrations of Stilbestrol

stilbene-quinone was formed by including manganese dioxide in the system of reactants. To ascertain if stilbestrol might be converted to the quinone form under the conditions of the experimentation reported herein, spectra of solutions of a stilbene-quinone prepared with manganese dioxide, and the experimental solution of stilbestrol in sodium hydroxide employed in these studies, were examined. The spectra of the two stilbestrol preparations are shown in Figure 11. The spectra of the experimental stilbestrol solution and the stilbene-quinone are quite similar in that they both show an absorption peak at approximately 250 mu. It was also observed that the stilbene-quinone solution had a yellow color which was similar to that observed developing in the stilbestrol solution on standing at room temperature. Fieser (7) also reported development of a yellow color on formation of stilbene-quinone. The data indicated that stilbestrol appears to be capable of existing in the quinone form under the conditions of this experimentation. If this is true then these two materials should behave similarly in an experimental system.

To elucidate the activity of stilbene-quinone in the experimental system stilbestrol solutions which were aged for varying time periods and the prepared quinone were studied simultaneously. The stilbestrol solutions and the prepared quinone were added to identical test systems and the rate of TPN^+ reduction was followed employing glucose-6-phosphate as the substrate. Results (Figure 12) indicated that the stilbene-quinone demonstrated an inhibitory activity similar to that observed in the test system containing the aged stilbestrol solution. The fresh stilbestrol solution also inhibited TPN^+ reduction, but to a lesser extent than the stilbene-quinone and aged solution. These findings suggest

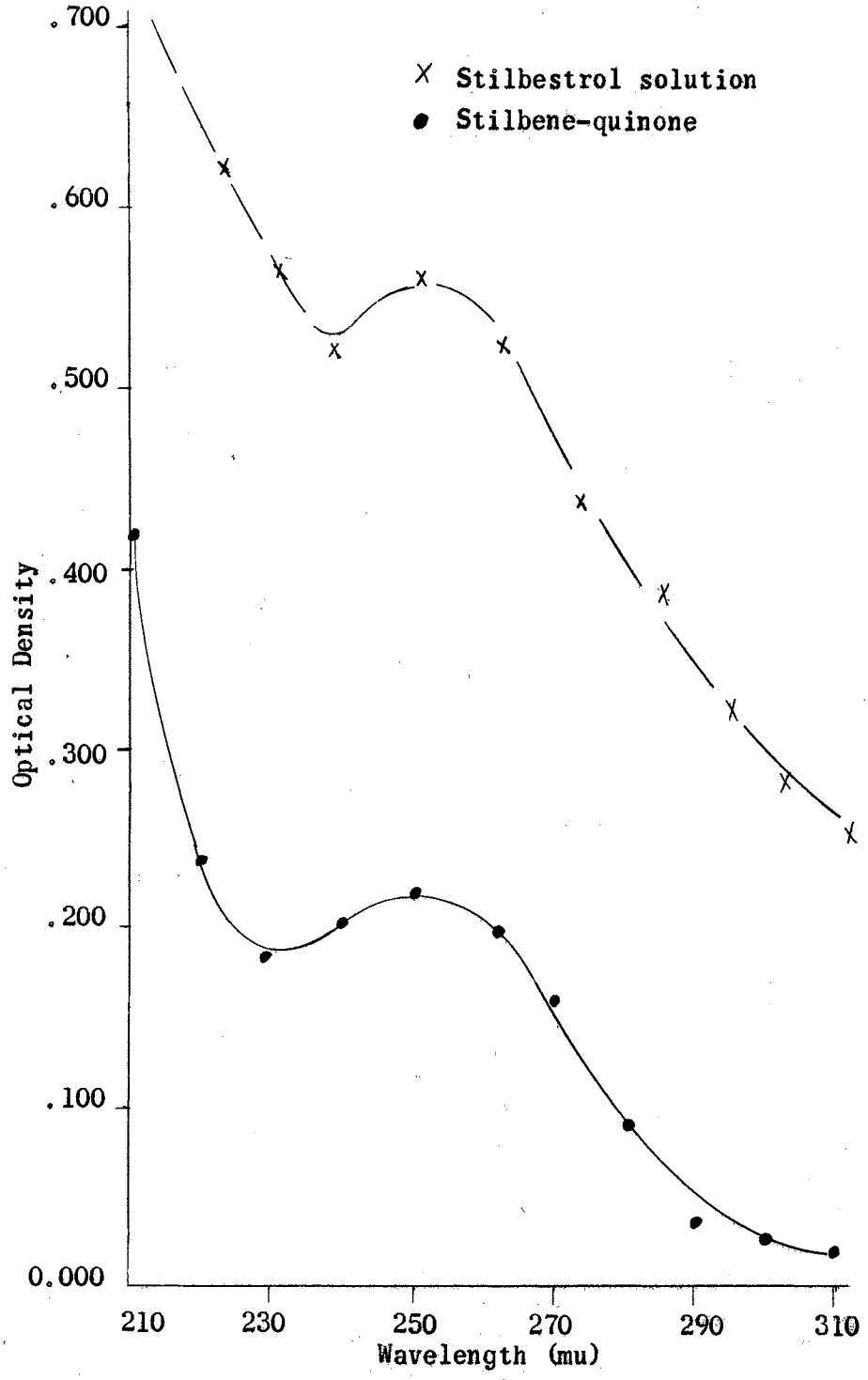

Figure 11. Absorption Spectra of a Stilbestrol Solution Used in Experimentation and a Manganese Dioxide Prepared Stilbene-Quinone

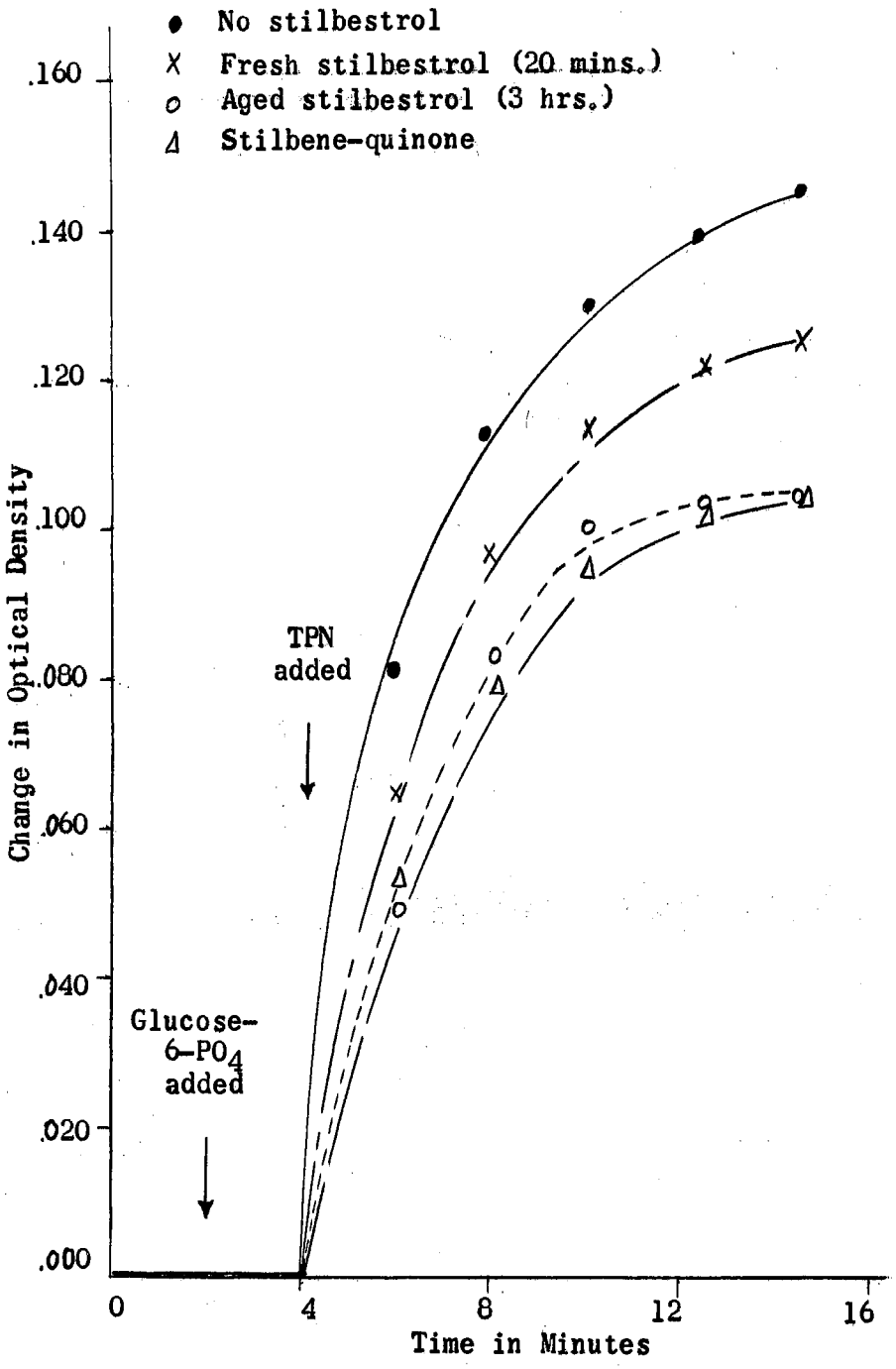

Figure 12. The Reduction of Triphosphopyridine Nucleotide by Glucose-6-phosphate Dehydrogenase in the Presence and Absence of Freshly Prepared and Aged Stilbestrol Solutions and Stilbene-Quinone

that stilbestrol may affect metabolic reactions by influencing electron transport. The evidence suggests that the estrogen is serving as a hydrogen carrier, since suppression of rate and extent of TPN^+ reduction was observed in the presence of stilbestrol.

CHAPTER IV

SUMMARY AND CONCLUSION

This study was undertaken to elucidate the effect of stilbestrol on metabolic processes of A. aerogenes. Results of the experimentation reported herein show that stilbestrol exerts a stimulatory effect on the growth of A. aerogenes in a synthetic salts medium containing starch. Additional experimentation indicated that the rate of raffinose oxidation by this organism is stimulated in the presence of the synthetic estrogen. The test organism was not capable of utilizing stilbestrol as a source of carbon and energy. In opposition to the stimulatory trends were the observations which indicated that stilbestrol inhibited the oxidation of maltose, fructose, glucose, glutamate, oxalacetate, succinate, and pyruvate. These inhibitions were observed in oxidative reactions of intact cells, cell-free extracts, and protein fractions of A. aerogenes.

The experimental data indicate that the activity of stilbestrol could be correlated to the estrogen concentration, preexposure of the cells to stilbestrol, and the pH of the reaction system. Results suggested that these variables influenced the system by allowing higher concentrations of stilbestrol to reach the sensitive sites. Stilbestrol did not appear to exert a bactericidal effect.

Additional experimentation indicated that the stilbestrol inhibition of glucose, succinate, or pyruvate oxidation by intact cells or cell-free

extracts, could not be reversed by the incorporation of a variety of compounds which are active in electron transport. In addition, the inclusion of various organic metabolites and metallic ions did not result in reversal of the suppressed oxidation. Experimental evidence was also obtained which indicates that stilbestrol does not function in transhydrogenation reactions in A. aerogenes.

These results appear to be conflicting and contradictory. However, the observation that stilbestrol may exist in the quinone form, under the conditions of the experimentation reported in this investigation, allows explanation of these phenomena.

It is suggested that stilbestrol exerts its activity on the metabolism of A. aerogenes by serving as an electron carrier or trap. It is proposed that in substrate oxidations where oxygen consumption is enhanced or growth stimulated, stilbestrol is serving as a hydrogen donor to intracellular anabolic processes thereby resulting in the more rapid synthesis of cellular components. By serving as an additional electron donor to synthetic mechanisms, the rate of enzyme synthesis could be stimulated thereby accounting for the observed stimulatory effects. It is interesting to note that this effect was observed with substrates requiring a period of "adaptation." This interpretation is supported by the observation that the rate rather than the extent of oxygen consumption was influenced. The diversions of electrons to a stilbene-quinone rather than to oxygen would give a decreased rate of oxygen consumption, while the donation of these hydrogens to anabolic reactions could result in increased cellular activity, thus the total oxygen consumed would not necessarily be affected.

Similar explanations may be applied to the results obtained with ammonium sulfate fractionated protein solutions of A. aerogenes. Results

indicated that TPN^+ reduction was decreased in the presence of stilbestrol during glucose-6-phosphate and isocitrate oxidation. The fractionated protein solutions required the addition of electron carriers in order for substrate oxidation to occur, which indicated that the electron transport systems had either been removed or were present in ineffective quantities. It is also reasonable to assume that all of the components required for anabolic reactions to proceed were not present. In the absence of cellular electron transport systems and synthetic mechanisms, it is suggested that stilbestrol may serve as an electron donor for oxygen or an intermediate carrier to oxygen, or as an electron trap. This explanation would apply to the decrease in TPN^+ reduction in the presence of stilbestrol, as the quinone form may be accepting hydrogen that would otherwise go to TPN^+, or may be oxidizing TPN·H as it is formed but at a slower rate thus accounting for the decrease in optical density change. The unknown acceptor of electrons from stilbestrol is probably limiting in this system, since all concentrations inhibit to about the same extent though not at the same rate.

Experimental results have not yet been obtained which confirm these suggestions, since a satisfactory method for following the oxidation and reduction of stilbestrol has not been devised. However, the author suggests that the experimental evidence included in this report substantiates the view that stilbestrol is functional as an intracellular hydrogen carrier. The site and mechanisms of stilbestrol activity in the electron transport system remains to be elucidated as to the sites and mechanisms of stilbestrol activity in anabolic processes.

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A P P E N D I X

Results of plate counts which were performed to confirm the results obtained by measuring the growth rate of A. aerogenes in the presence and absence of stilbestrol, employing various carbohydrates as sources of carbon and energy.

TABLE V.

TOTAL NUMBER OF VIABLE CELLS IN A SYNTHETIC MEDIUM CONTAINING GLUCOSE AND GLUCOSE PLUS STILBESTROL

Time in Hours	Glucose	Glucose + Stilbestrol
0	5.2	
2	12.6	10.1
4	60.0	70.0
6	170.0	170.0

All counts x 10^6

TABLE VI

TOTAL NUMBER OF VIABLE CELLS IN A SYNTHETIC MEDIUM CONTAINING MALTOSE AND MALTOSE PLUS STILBESTROL

Time in Hours	Maltose	Maltose + Stilbestrol
0	0.2	
3	1.1	0.9
5	4.7	4.8
8	67.0	60.0
11	90.0	95.0

All counts x 10^7

TABLE VII

TOTAL NUMBER OF VIABLE CELLS IN A SYNTHETIC MEDIUM CONTAINING RAFFINOSE AND RAFFINOSE PLUS STILBESTROL

Time in Hours	Raffinose	Raffinose + Stilbestrol
0	0.3	
2	0.6	0.5
4	12.6	12.7
8	88.0	81.0
24	138.0	127.0

All counts x 10^7

TABLE VIII

TOTAL NUMBER OF VIABLE CELLS IN A SYNTHETIC MEDIUM CONTAINING STARCH AND STARCH PLUS STILBESTROL

Time in Hours	Starch	Starch + Stilbestrol
0	0.3	
24	0.9	10.5
41	2.0	190.0
46	148.0	359.0
49	190.0	430.0
55	430.0	400.0

All counts x 10^7

VITA

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Master of Science

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